

Induction of a cell wall variant of the gonococcus by human amylase

A MELLERSH, S HAFIZ, M G McENTEGART, AND I GEARY

From the Department of Medical Microbiology, University of Sheffield Medical School, Sheffield

SUMMARY Human amylase has been shown to be an effective and powerful inhibitor of the gonococcus in vitro. Its action appears to be on the bacterial cell wall. When tested in osmotically stabilised liquid media the organism was not inhibited; however the organisms which grew in such cultures appeared to be cell wall variants, which were less sensitive to penicillin than the parent strains. Studies on these variants suggest that they are "transitional-phase variants." Since cervical mucus has much in common with an alkaline osmotically stabilised liquid medium and also contains a high concentration of amylase, it seems possible that these variants may occur in vivo.

Introduction

Normal human saliva is a potent and specific inhibitor of the gonococcus in vitro, and this effect has been shown to be due to amylase.¹ High concentrations of amylase are found in saliva, cervical mucus,² and seminal fluid,³ and yet the gonococcus appears to survive in areas bathed by these fluids.

Because of its high molecular weight, about 50 000 daltons, it seems probable that amylase acts at the level of the outer cell wall and does not penetrate the bacterium. Inhibitors of bacteria that act at the level of the cell wall are known to induce cell-wall-defective, osmotically fragile variants.⁴

A major difference between growth of the gonococcus in vivo and in vitro is that in the former the body supplies a degree of osmotic stability; we felt that this protection—of an osmotically fragile, cell-wall-defective variant—might explain the survival of the gonococcus in the areas containing high concentrations of amylase.

Materials and methods

MEDIA

The liquid medium used was ANM,⁵ and this was osmotically stabilised by the addition of 1% polyvinylpyrrolidone (BDH, Analar; molecular

weight, 700 000); this is referred to as the liquid medium. Subcultures were made on to plates of Difco GC agar base with 2% defined supplement. When plates were to be osmotically stabilised 1% polyvinylpyrrolidone (BDH) was added. Subcultures from the liquid medium were made on both osmotically stabilised (OS) plates and non-stabilised (NOS) plates. Normally, ANM has a pH of 7.2, but some of the liquid media was adjusted with sodium hydroxide, (1 mol/l) to a pH of 8.0 (the pH of the cervical mucus of a woman on the contraceptive pill).

N GONORRHOEAE STRAINS

International strain WHO VII, β -lactamase producers HKI and RI, as well as recent isolates from the Sheffield Special Clinic, were used for this study.

AMYLASE EXTRACTION

Amylase was extracted from pooled human saliva by the method of Meyer *et al.*⁶ Purified amylase was added to the liquid media to give a final amylase activity of approximately 2000 Somogyi units per ml.

STAINING

Cultures were centrifuged at 2000 rev/min for 10 minutes and the deposit washed with phosphate-buffered saline, placed on slides, and stained using Dienes or Gram stain.

MILLIPORE FILTRATION

Eighteen-hour cultures in stabilised liquid medium were passed through 0.45 μ Millipore filters and the filtrate cultured in stabilised liquid medium as well as on OS and NOS plates.

Address for reprints: Professor M G McEntegart, Department of Medical Microbiology, University of Sheffield Medical School, Beech Hill Road, Sheffield S10 2RX

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IMMUNOFLUORESCENCE

A loopful of suspension prepared from the centrifuged deposit was allowed to dry in air on a standard microscope slide, fixed in acetone, and covered with a 1/16 dilution of antigonococcal immunofluorescent conjugate (Bacto-FA *N gonorrhoeae* Difco). Slides were left in a moist box for 15 minutes, washed in distilled water, dried, and mounted in glycerol buffer for examination.

ELECTRON MICROSCOPY

Centrifuged deposits were suspended in 3% buffered glutaraldehyde for one hour and then post-fixed with 1% osmium tetroxide for 30 minutes, prestained with uranyl acetate for 30 minutes, and dehydrated through increasing concentrations of ethyl alcohol to epoxy propane and finally embedded in epoxy resin. Ultra-thin sections were cut using a Reichert OMU 3 ultramicrotome. The thickness of sections ranged from 60 to 80 nm. The sections were mounted on copper grids and stained with Reynolds lead citrate.

ENDOTOXIN ASSAY (CHICK EMBRYO TESTS)

Eighteen-hour cultures of WHO VII strain gonococci grown in the liquid medium with amylase were centrifuged and the deposit resuspended in saline and standardised with an EEL nephelometer to contain 10^6 organisms per ml. For the test, 10-day-old embryonated eggs were used. These were kept in a humidified incubator at 37°C. For inoculation, a rectangular window (approximately 3×5 mm) was cut over a prominent allantoic vein (while candling) and the shell flap was lifted off with a needle. After the windows had been cut the eggs were undisturbed for one hour to minimise bleeding on inoculation; 0.1 ml of the suspensions was inoculated into each egg with a 27-gauge needle. Control eggs received a suspension of vegetative-phase bacteria at the same concentration or, as a negative control, phosphate-buffered saline. After inoculation, the eggs were returned to the incubator for two days and were candled at 24 and 48 hours. When death of the embryo was suspected, it was confirmed by immediate examination of the contents of the egg. Specific endotoxin deaths showed very characteristic pathological changes and usually occurred in the first 24 hours after inoculation.

PENICILLIN SENSITIVITY

Benzyl penicillin was diluted in the liquid medium to give a series of dilutions from 1.0 to 0.0025 units per ml as shown in the table. Amylase and 0.1 ml of an 18-hour culture of WHO VII strain gonococci (in the liquid medium with amylase) were added to each dilution. WHO VII strain gonococci grown without amylase and the Oxford *Staphylococcus* were added

TABLE Sensitivity of two strains of *N gonorrhoeae* and the Oxford *Staphylococcus* to benzyl penicillin

Organisms	Concentrations of penicillin (units per ml)					
	1.0	0.1	0.05	0.01	0.005	0.0025
<i>Staphylococcus</i> Oxford (control)	—	+	+	+	+	+
WHO VII vegetative phase (non-amylase treated)	—	—	—	—	+	+
WHO VII variant phase (amylase-treated)	—	+	+	+	+	+
+ Growth — no growth						

to two similar series of penicillin dilutions as controls. Penicillinase production was detected by the addition of a chromogenic cephalosporin (Nitrocefin, Glaxo).

Results

CULTURE GROWTH AND STAINING

In the osmotically stabilised liquid media, all strains showed visible growth in 18 hours. Subcultures made on OS and NOS plates at 18 hours all grew. Colonies were more numerous on OS plates, especially on subcultures made more than 48 hours after inoculation of the liquid media. On some occasions no growth was obtained on NOS plates after 48 hours. When examined in wet preparation the growth in the liquid medium was typically seen as clumps of organisms up to 2 mm in diameter. Growth in the medium at pH 8.0 was similar to that at 7.2. The colonial forms on primary subculture from the liquid medium on to solid medium were of three types. Most were typical gonococcal colonies. A varying number, up to 50%, were atypical with a raised circular centre and a flat periphery with a well defined border. Subsequent subcultures from these colonies yielded typical gonococcal colonies of Gram-negative diplococci which fermented glucose but not maltose or sucrose. About 1% of colonies were of the L form. Dienes-stained films of 18-hour cultures showed aggregates of weakly staining granular material with a few darker staining diplococci (fig 1). These aggregates were Gram-negative and, when tested with a known antigonococcal serum, showed bright on specific fluorescence (fig 2). Millipore filtration was unsuccessful in that only a few viable units passed through the filter.

ELECTRON MICROSCOPY

On electron microscopy the control organisms incubated without amylase (fig 3) had a uniform shape, typical cell wall structure, and an osmophilic cytoplasm indicating a large number of ribosomes.

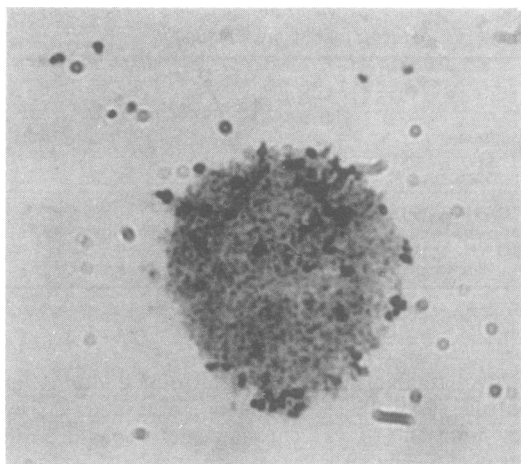


FIG 1 *Impression preparation of an L-form colony grown from a subculture of gonococci exposed to amylase in liquid medium for 18 hours. Dienes stain $\times 1230$.*

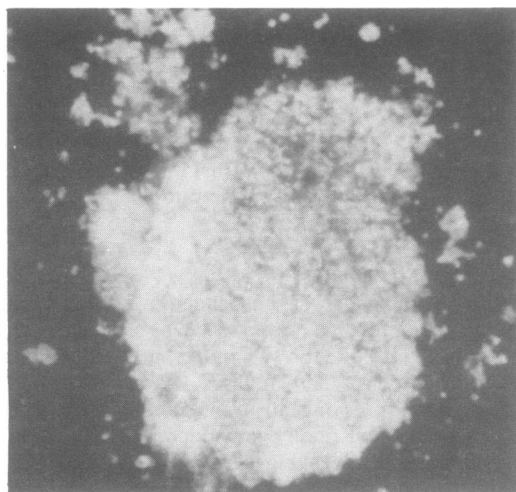


FIG 2 *Impression preparation of L-form colony showing bright fluorescence when stained with a specific antigenococcal conjugate. $\times 1230$.*

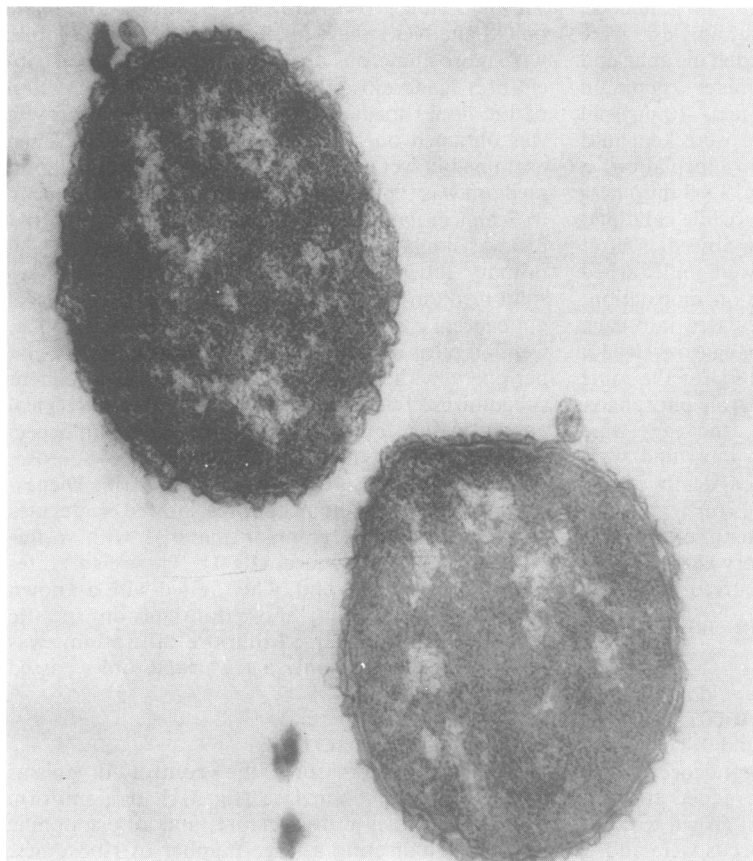
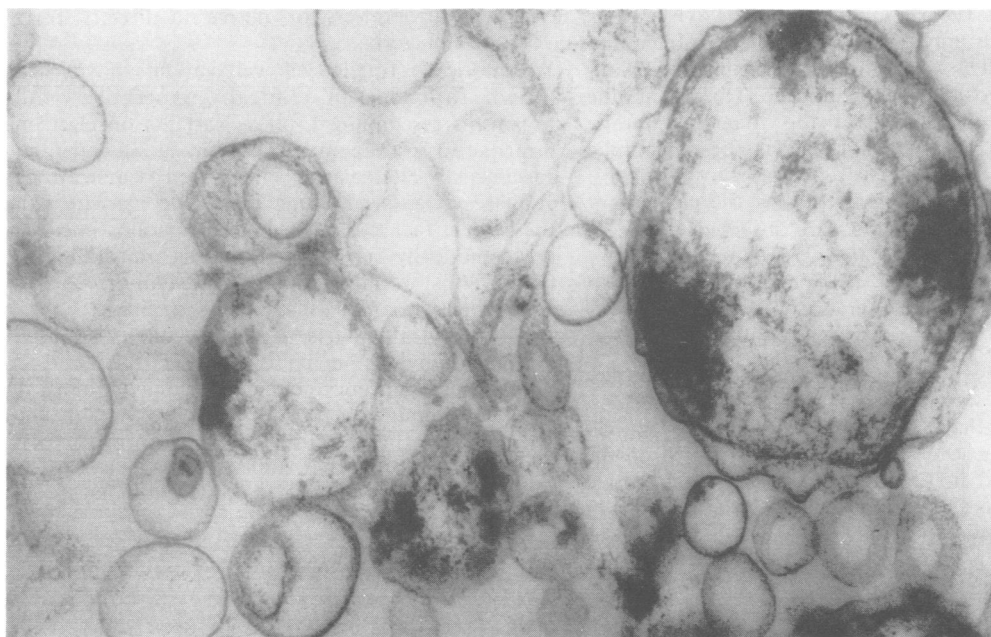
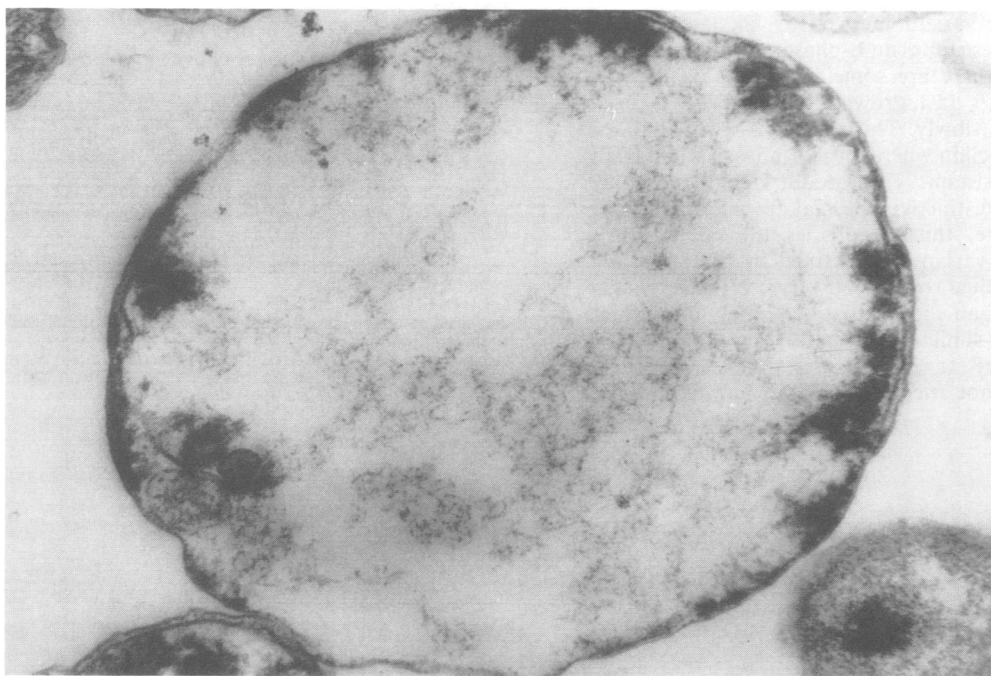


FIG 3 *Electron micrograph ($\times 54\,500$) showing normal appearance of control gonococcus.*



(a)



(b)

FIG 4 (a) and (b) *Electron micrographs ($\times 54\,500$) of amylase-treated gonococci showing gross variation in cell size and damage to the cell wall.*

They showed typical endotoxin blebs. Organisms incubated with amylase were irregular in shape and size (fig 4); they were limited by a simple cell wall, often just a cytoplasmic membrane. This membrane was often duplicated and the inner membrane discontinuous. In larger organisms the osmophilic material was less and scattered at the periphery. Numerous smaller units resembling the elementary bodies of L-phase variants were observed, but these were presumably not viable, as viable units were rarely obtained after Millipore filtration. No endotoxin blebs were observed.

ENDOTOXIN ASSAY

The organisms grown in the liquid medium containing amylase were toxic to chick embryos (70% endotoxic deaths) but not as toxic as the vegetative-phase bacteria (90% endotoxic deaths). No deaths were observed in the controls. (Each of the three groups consisted of 30 chick embryos.) The growth in the liquid media with amylase was still sensitive to penicillin but not as sensitive as the vegetative-phase bacteria (table). β -lactamase-producing bacteria continued to produce β -lactamase throughout the experiments.

Discussion

Although the variant induced by amylase in these experiments resembles an L-phase variant on electron microscopy, there are some important differences. This variant is fast growing whereas the L-phase variant grows slowly. This variant is sensitive to the action of penicillin whereas the L-phase variant is, by definition, resistant. This variant cannot reproduce serially in a distinctive colonial form on agar. We feel, therefore, that it satisfies the criteria of a "transitional variant" as defined by McGee *et al.*⁷ Ota *et al.*⁸ studied reverting L-phase variants on agar and observed an intermediate colonial form which was strikingly similar to those forms we observed on subculture of these variants. However, these workers⁸ did not attempt to grow it in liquid media,

study its properties, or derive it directly from vegetative bacteria.

Osmotically fragile cell wall variants have been shown to occur in clinical gonorrhoea⁹ and organisms resembling L-phase variants on electron microscopy have been observed in clinical specimens.¹⁰ These variants have been assumed to be L-phase variants, although this is inconsistent with the clinical fact that penicillin cures gonorrhoea. If the osmotically fragile fraction was transitional in nature, this would be more consistent, since this transitional variant is sensitive to penicillin.

The cervical mucus is osmotically stabilised, contains amylase in high concentration, and is alkaline. Attempting to simulate these conditions in vitro, we obtained a transitional-phase variant, and we consider therefore that it is likely that these variants occur in vivo and may have a part in explaining the relatively asymptomatic nature of infections of the uterine cervix.

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